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SUB-ACUTE TOXICITY AND GENOTOXICITY ASSESSMENT OF THE RHIZOME EXTRACT OF *ACORUS CALAMUS* L., A MEDICINAL PLANT OF INDIA

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ABSTRACT

The rhizomes of Acorus calamus L. have been used as a folk medicine against many diseases and disorders in India. This study assessed the sub-acute toxicity and genotoxicity potentials of *A. calamus* rhizome extract in Swiss albino mice. The mice were treated with a low (400 mg/kg) and a high (800 mg/kg) dose of *A. calamus* rhizome extract for 14 days. The sub-acute toxicity study analyzed the haematological, biochemical parameters, and histopathology of liver, kidney and spleen of animals. The genotoxicity assessement was done in vivo by chromosomal aberrations assay on bone marrow cells of mice. No mortality or behavioural adverse effects were observed in animals by 800 mg/kg oral dose of extract for 14 days. In biochemical assay, only 800 mg/kg dose revealed significant increase in alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total and direct bilirubins. No major effects were evident in any biochemical parameters by 400 mg/kg dose. In the haematological assay, only the high dose of extract showed significant increase in the numbers of neutrophils and lymphocytes, however, all blood parameters appeared normal in mice treated with 400 mg/kg dose of extract. The histopathological assessments of liver, kidney and spleen tissues also showed several deviations from their normal architectures only in the high dose treated group of animals. The genotoxic assessments revealed maximum frequencies of chromosomal aberrations in 800 mg/kg extract-treated animals. Taken together, this study suggested that 800 mg/kg dose of *A. calamus* rhizome extract may cause several toxic and genotoxic effects in experimental animals.

KEYWORDS: Acorus calamus, folk medicine, genotoxicity, helminth parasite, histopathology, sub-acute toxicity.

INTRODUCTION

Many indigenous communities in the world use medicinal plants for treating various ailments, which are mainly based on traditional knowledge or beliefs of people. In India, about 7,000-7,500 species of plants have medicinal usage in folk and documented systems of medicine.[1] It is believed that as many as 25,000 different formulations are prepared from these plants as a cure against various diseases and disorders.[1] Therefore, owing to the ever growing popularity of herbal medicines world-over, especially in Asian and African regions, long-term systematic studies seem desirable not only to validate their acclaimed effects, but also to investigate their safety credentials.

Acorus calamus L. (Acoraceae), popularly known as Sweet flag, is a perennial herb which prefers to grow in semi-aquatic habitats. It is commonly distributed in the northern temperate and sub-tropical regions of Asia, North America, and Europe.[2] In India, this plant usually grows at an altitude of 2200 m in the Himalayan region. In different cultures of the world, *A. calamus* is regarded as one of the important medicinal plants to cure a variety of ailments.[2,3] In particular, it finds important ethnomedicinal uses in the Indian and Chinese traditional medicines.[2] In the Ayurvedic medicine, *A. calamus* rhizomes are believed to be beneficial for emetic, expectorant, aphrodisiac, antimicrobial, laxative, diuretic, antispasmodic, carminative and anthelmintic properties.[2,4] This plant has also been found to have profound central nervous system (CNS) actions, such as anticonvulsant, sedative, hypnotic, tranquilizing, and memory enhancing properties, which justifies its use in some CNS diseases in Ayurvedic medicine.[3] Some experimental studies also support use of this plant against human intestinal helminthic infections.[5-8] The phytochemical screening of *A. calamus* has revealed that it possesses glycosides, flavonoids, saponins, tannins, as the main secondary metabolites.[9-11] whereas, methyl eugenol, cis-methylisoeugenol, α and β-asarone, geranylacetate, shyobunone, epishyobunone, isoshyobunone, calamenene, asaronaldehyde, acorenone, etc. constitute as some of the key chemical compounds.[12-14] Interestingly, the proportions of many of its chemical compounds, especially its active principle β-asarone, are known to vary in its different varieties

(diploid, triploid, tetraploid, etc.) that grow in different geographical regions.[15,16] It is therefore hypothesized that *A. calamus* may possess different levels of therapeutic efficacy owing to the differences in the amounts of its active principle β-asarone present in different geographical regions.[12,13] In a previous study,[14] conducted on *in vivo* anthelmintic effects of rhizomes of this plant in *Hymenolepis diminuta*-rat experimental model, it was found that *A. calamus*, collected from Tripura (India), possesses significant anthelmintic effects. This study also established that the local variety of *A. calamus* in Northeast India is tetraploid and the active fraction of its rhizomes contains a very high amount (83.54%, w/w) of β-asarone. It would thus appear from the aforesaid discussion that the amount of β-asarone in *A. calamus* is of paramount importance, as it may significantly affect the perceived therapeutic efficacy of this plant in a geographical area.[14,15] Moreover, from time to time, questions have also been raised about the possible toxicological risks associated with the use of *A. calamus*, as this plant follows a geographical pattern of distribution with respect to ploidy and its β-asarone content.[16,17] Recently, some workers have suggested that since rhizomes of *A. calamus* has been used in India for thousands of years, without reports of cancer, it may be safe to use this plant.[3] However, these claims need to be verified scientifically as no reliable data are available to conclusively comment anything about the sub-acute toxic and genotoxic effects of *A. calamus* rhizomes.

It is in the light of this background that the present study was undertaken to assess the sub-acute and genotoxic effects of *A. calamus* rhizome extract in Swiss albino mice, which discusses the effects of two different doses, a low dose (400 mg/kg) and a high dose (800 mg/kg), of *A. calamus* rhizome extract on some biochemical, haematological and organ histopathological parameters of mice, besides *in vivo* genotoxicity assessment in mice using chromosomal aberration assay.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study were of standard analytical grade and obtained from the respective sources: colchicine (Hi-Media), beta-asarone (Sigma), methanol (Merck), giemsa stain (Hi-Media), haematoxylin (Hi-Media).

Experimental Animals

Healthy Swiss albino mice of either sex, weighing 25-30 g, were used. The animals were maintained individually in acrylic cages under the standard laboratory conditions and had *ad libitum* access to food and water. All experiment involving animals were approved by the Institutional Ethics Committee (Animal Models) of North-Eastern Hill University (NEHU), Shillong.

Plant Material

The rhizomes of *A. calamus* were collected from North Tripura district of Tripura, India in October, 2010. The plant material was identified by a plant taxonomist in the Department of Botany, NEHU, Shillong. A voucher specimen (No. AKY-11883) of studied material has been retained in the Department of Zoology, NEHU. The rhizomes were air-dried under shade, powdered and extracted with methanol using a Soxhlet extractor, as mentioned in our previous study.[14] The final yield of methanol crude extract was 15% (w/w). The rhizome extract of plant used in this study has been previously characterized and its anthelmintic active fraction contained 83.54% (w/w) of β-asarone.[14]

Sub-acute Toxicity Study

The sub-acute toxicity assays were performed as per the Organization for Economic Co-operation and Development (OECD) guideline 407,[18] with slight modifications. Based on our previous findings of the acute toxicity test [148], two different doses of rhizome extract, i.e. 400 mg/kg (low dose) and 800 mg/kg (high dose), were selected and administered orally once daily for 14 days to two different groups of mice (n = 10). The third group of mice (n = 10) served as control. After extract treatments, all the animals were observed daily for any abnormal behavioural adverse effects or mortality for 14 days. At the end of treatment (the 15th day), all the animals were anaesthetized, and their blood samples were collected through cardiac puncture with and without anticoagulant (EDTA), for the analysis of haematological and biochemical parameters, respectively. In haematological assay, red blood cell (RBC), white blood cell (WBC) and platelet counts, haemoglobin, and mean corpuscular haemoglobin concentration were determined using a haematology analyzer (Nihon Kohden Celltac MEK 6410 K Cell Counter). For biochemical analysis, blood without additive was centrifuged at 3000 × g at 4°C for 10 min, serum was separated and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, total bilirubin, direct and indirect bilirubin, urea and creatinine were estimated using a Semi-automated Biochemical Analyzer (Bayer RA-50). For histopathological assessments, the liver, kidney and spleen of animals were excised, rinsed in physiological saline and preserved in 10% buffered saline. Tissue sections were cut of 5-8 µm thickness and stained with haematoxylin and eosin. Histopathological observations were made using a Leica DFC425C light microscope and representative photomicrographs were obtained using a digital camera.

Genotoxic Study

The genotoxic effects were studied using chromosomal aberration assay in bone marrow cells of Swiss albino mice. Fifteen animals were divided into 3 groups. Group I of animals served as control and received only normal saline, while group II and group III of animals were administered orally with 400 mg/kg and 800 mg/kg of *A.*

calamus rhizome extract respectively, given orally once daily for a period of 14 days. At the end of the treatment, animals of all groups were subjected to mitotic arrest, which was initiated 2 h prior to sacrifice by an intra-peritoneal injection of colchicine (4 mg/kg). Bone marrow metaphase chromosome spreads were made according to the methods described by Sharma and Sharma.[19] One hundred well-spread metaphase plates were examined per animal for scoring the chromosomal aberrations, including chromatid breaks (CB), gaps, isochromatid break (ICB), chromosomal fragments (CF), exchanges (EXCH) and sister chromatid unions (SCU). Gaps have not been considered here for the statistical analysis of chromosomal aberrations incidences, as their genetic significance has been considered as controversial by the World Health Organization.[20]

Statistical analysis

Data are expressed as mean±standard errors of the mean (S.E.M.). Statistical analysis was done using Student's t-test, and by one-way analysis of variance, followed by Bonferroni test. P≤0.05 was considered as statistically significant.

RESULTS

Sub-acute Toxicity Analysis

No mortality or behavioural adverse effects were observed in the sub-acute toxicity studies during the entire experiment period of 14 days. The data regarding the effects of *A. calamus* rhizome extract on biochemical parameters of mice are given in Table 1. Only in high dose (800 mg/kg), the extract caused a significant increase in the level of liver enzymes, alanine aminotransferase (p≤0.01), aspartate aminotransferase (p≤0.05), and total and direct bilirubins (p≤0.05). Also, alkaline phosphatase showed a significant reduction (p≤0.05) in both the extract treated groups. As such, the lower dose of extract (400 mg/kg) did not reveal any noticeable changes, except for a slight rise in total and direct bilirubins (p≤0.05).

Table 1: Effects of oral administration of *A. calamus* rhizome extract for two weeks on some biochemical parameters of mice (n=10).

Parameters	Control	Plant extract	
		400 mg/kg	800 mg/kg
AST (U/L)	93.83± 9.04	95.00±4.40	160.30±1.47*
ALT (U/L)	49.67±8.16	52.40±0.87	140.00±0.93**
ALP (U/L)	256.33±8.38	212.30±2.36*	180.40±1.69*
Total bilirubin (mg/dL)	1.21±0.20	0.74±0.00*	1.94±0.01*
Direct bilirubin (mg/dL)	0.16 ±0.05	0.66±0.01**	0.72±0.00*
Indirect bilirubin (mg/dL)	0.44 ±0.07	0.29±0.01*	0.35±0.00*
Urea (mg/dL)	13.00 ±0.25	22.00±0.52	23.80±0.65*
Creatinine (mg/dL)	0.52 ±0.04	0.51±0.06	0.53±0.05

Data are expressed as mean±SEM, *p≤0.05 versus control, one-way ANOVA post-hoc Bonferroni test; **p≤0.01 versus control, one-way ANOVA post-hoc Bonferroni test. SEM: Standard error of the mean, ANOVA: Analysis of variance, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, A.calamus: *Acorus calamus*.

In the haematological parameters, only the highest dose of extract (800 mg/kg) showed significant increase (p≤0.05) in the levels of neutrophils and lymphocytes (Table 2). Interestingly, as compared to control, all the haematological parameters were found to be normal in animals treated with 400 mg/kg dose of plant extract (Table 2).

Table 2: Effects of oral administration of *A. calamus* rhizome extract for two weeks on some haematological parameters of mice (n=10).

Parameters	Control	Plant extract	
		400 mg/kg	800 mg/kg
RBC (mL/cumm)	3.24±0.05	3.45±0.05	4.31±0.05*
WBC (mL/cumm)	4.40±0.06	4.45±0.06	3.37±0.05*
Neutrophils (%)	40.10±0.38	41.6±0.85	51.90±0.53*
Lymphocytes (%)	52.40±0.67	50.5±0.78	68.50±0.69*
Monocytes (%)	2.30±0.30	2.30±0.37	2.30±0.37
Haemoglobin (g/dL)	10.30±0.06	11.63±0.20	10.59±0.25
Mean corpuscular Hb conc (g/dL)	31.14±0.50	30.04±0.46	31.72±0.61
Platelet count (10^3/µl)	1.40±0.11	1.50±0.29	2.68±0.18**

Data are expressed as mean±SEM. *p≤0.05 versus control, one-way ANOVA post-hoc Bonferroni test; **p≤0.01 versus control, one-way ANOVA post-hoc Bonferroni test. SEM: Standard error of the mean,

ANOVA: Analysis of variance, RBC: Red blood cell, WBC: White blood cell, Hb: Haemoglobin, *A. calamus*: *Acorus calamus*.

The histological features of the liver from control mice showed normal features, without any noticeable alterations (Figure 1). The histological analysis of liver, kidney and spleen tissues of mice from 400 mg/kg extract-treated group also did not reveal any abnormal features (photomicrographs not shown). In contrast, the histological analysis of liver of animals treated with 800 mg/kg dose of extract showed histopathological alterations, such as dilation of central vein indicating backflow of circulation, leucocytic infiltration, apoptosis and nuclear pyknosis (Figures 1b, c, d). Similarly, the histological analysis of kidney from control mice revealed normal architecture and the architectures of glomeruli, and the distal and proximal tubules appeared preserved (Figures 2a, c, e). However, administration of *A. calamus* extract at 800 mg/kg dose for two weeks showed significant alterations in about 85% of the treated mice, in the form of dilatation of sub-capsular space of glomeruli and formation of vacuoles surrounding capsular and distorted renal tubules (Figures 2b, d, f). The spleen histology of mice from the control group appeared with normal architecture, i.e., displaying a well-conserved white pulp (Figure 3a). However, the histological analysis of spleen from mice treated with 800 mg/kg dose of *A. calamus* extract showed reduction in the size and vacuole formation in the white pulp region (Figures 3b, d).

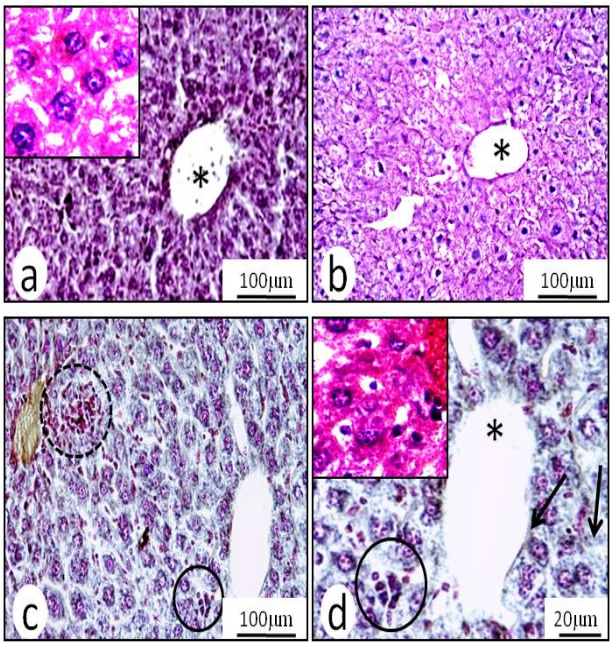

Figure 1: (a) Cross section of the liver from control mouse, showing normal features and conserved central vein (*). (b-d) Cross section of the liver from mice treated with 800 mg/kg dose of *A. calamus* rhizome extract for two weeks, showing: (b) dilation of central vein (*), (c), leucocytic infiltration (dotted circle) and apoptotic nuclei (solid circle), (d) apoptotic nuclei in higher magnification (encircled) and pyknotic nuclei (regular arrow).

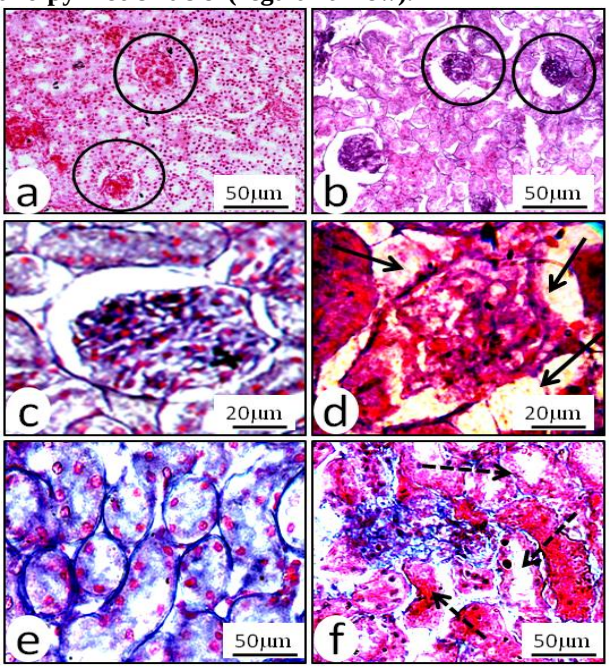

Figure 2: (a, c, e) Cross section of the kidney from control mouse, showing (a) the normal glomerulus (encircled area), (c) the normal glomerulus in higher magnification, (e) the normal renal tubules. (b, d, f,). Cross section of the kidney from mouse treated with 800 mg/kg dose of *A. calamus* rhizome extract for two weeks, showing (b) dilatation of sub-capsular space (encircled area) of glomeruli, (d) vacuoles (regular arrows) surrounding the capsular wall, (f) distorted renal tubules (dotted arrows).

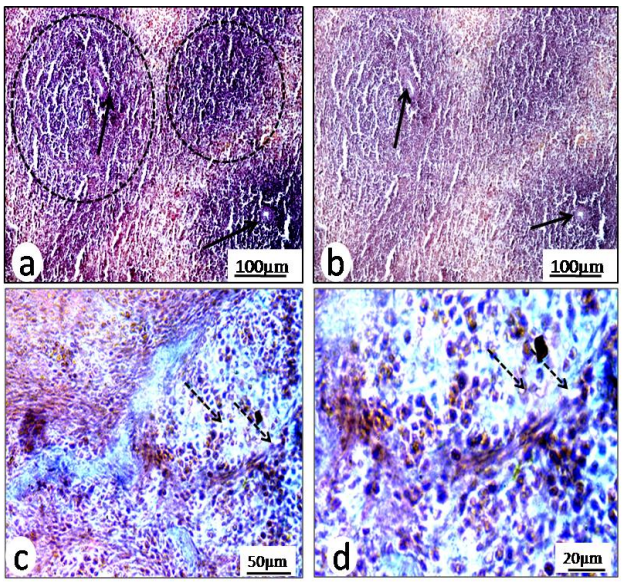

Figure 3: (a) Cross section of the spleen from control mouse, showing normal features, white pulp (dotted area) and central artery regular arrow. (b-d) Cross section of the spleen from mouse treated with 800 mg/dose of *A. calamus* extract for two weeks, showing: (b) decrease in size of white pulp area (solid arrow), (c) vacuolization in white pulp area (dotted

arrow), (d) vacuolization in white pulp area in higher magnification (dotted arrows).

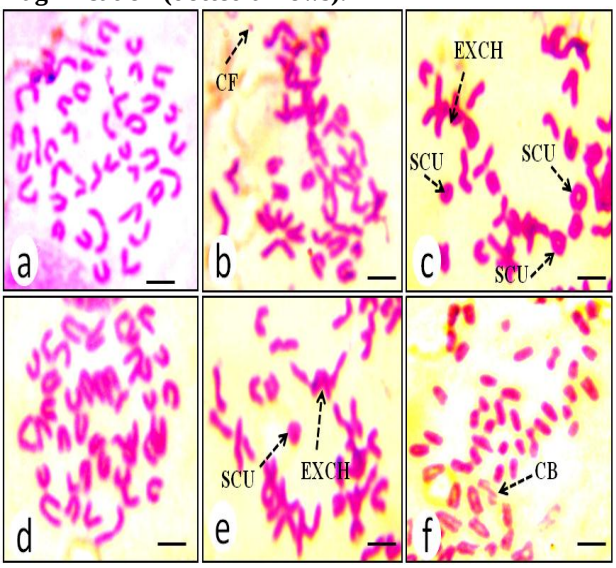

Figure 4: (a, d) Photomicrographs of bone marrow metaphase chromosome spreads of mice showing normal set of chromosomes.(b, c) showing chromosomal fragments (CF), sister chromatid unions (SCU) and exchanges (Exch) after treatment with *A. calamus* rhizome extract, (e, f) showing exchanges (Exch), sister chromatid unions (SCU) and chromatid breaks (CB) after treatment with β-asarone. Scale bar = 5 µm (*A. calamus* extract, 400 mg/kg and 800 mg/kg treatments were given orally to mice (n=5), once daily, for a period of 14 days).

Genotoxicity Analysis

The results of genotoxic testing of *A. calamus* rhizome extract (400 mg/kg and 800 mg/kg doses) in mice are presented in Figure 4 and Table 3. Metaphase analysis of the bone marrow cells from extract treated group showed various types of chromosomal aberrations (CA) such as, sister chromatid unions (SCU), chromosomal fragments (CF), exchanges (EXCH), chromatid breaks (CB) and isochromatid breaks (ICB) (Figures 4b, c, e, f). Of these, CB, EXCH and SCU were more frequent than other types of aberrations (Table 3). The maximum frequencies of various CA were evident in 800 mg/kg extract treated group of animals. The results also showed that the tested doses of *A. calamus* extract induced a statistically significant ($p \leq 0.001$) increase in the percentage of total aberrations even after excluding the gaps.

Table 3: Percentage frequency[a] of chromosomal aberrations in the bone marrow cells of mice after treatment with rhizome extract of *A. calamus* for two weeks (n=5).

Treatment	Mean aberrant metaphases (%)	Chromosome aberrations					Total (Mean±S.E.M)
		CB	ICB	CF	EXCH	SCU	
Control	1	0.9	0.4	0.2	0.00	0.00	0.30±0.17
Plant extracts							
400 mg/kg	2.5	2.0	0.2	0.4	1.1	1.0	0.94 ±0.32*
800 mg/kg	8.0	8.0	1.5	0.3	1.4	1.2	2.48 ±1.30**

Data are expressed as mean±SEM, [a]One hundred metaphases analyzed per animal, * $p \leq 0.05$ versus control, one-way ANOVA post-hoc Bonferroni test; ** $p \leq 0.001$ versus control, one-way ANOVA post-hoc Bonferroni test. SEM: Standard error of the mean, ANOVA: Analysis of variance, CB: chromatid breaks; ICB: isochromatid breaks; CF: chromosomal fragments, EXCH: exchanges, SCU: sister chromatid unions, *A. calamus*: *Acorus calamus*.

DISCUSSION

The use of folk medicines is very popular in many regions of Northeast India. Unlike other systems of Indian traditional medicine, folk medicines in India are not yet formally codified and their formulations are mostly employed without taking into account their toxicity aspects. There is a growing body of evidence now that reveal many herbal medicines if used in their irregular or high dose may cause various kinds of toxicity to their users.[21] For example, a traditional anthelmintic prepared from Maklua berries (Diospyros mollis) and used in Thailand has been found to be toxic in mice and known to cause temporary or permanent blindness.[22] In a similar manner, the latex of some species of Ficus has been traditionally used as vermifuge in Central and South America. However, due to its high toxicity, like severe hemorrhagic enteritis, this plant has been found unsuitable as a safe traditional anthelmintic.[23] It may be mentioned here that most of the toxicological studies on medicinal plants report that toxic effects due to the use of medicinal plants are primarily associated with hepatotoxicity, although adverse effects on other body organs such as kidney and/or genotoxic effects have also been documented[21] In addition, there are many other reports of allergic or toxic reactions related to the pharmacological actions of medicinal plants[24] Considering the aforesaid, it becomes increasingly necessary to have a thorough investigation on safety profiles of medicinal plants, which are used in traditional or folk medicines.

In the present study, we analyzed the sub-acute toxicity and genotoxic effects of *A. calamus* rhizome extract in mice. The results of this study clearly indicated that administration of high doses (800 mg/kg) of *A. calamus* extract to mice for 14 days does reveal many sub-acute and genotoxic effects in animals. Treatment with 800 mg/kg dose of extract showed increased levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin whereas, treatment with low dose did not produced any noticeable adverse effects. The liver function tests are monitored by measuring the levels of ALT and AST. The ALT is a cytoplasmic enzyme which is found in a very high concentration in the liver.[25,26] The increase of ALT in the serum indicates an evidence of hepatocellular damage.[27] The level of ALT increases when the hepatic cellular permeability is changed or if there is a necrosis and cellular injury.[28] Therefore, the present finding of an increase in ALT following treatment with *A. calamus* extract suggests some possible hepatocellular damage and hepatotoxicity due to the administration of extract to animals. The renal function tests are monitored by monitoring the levels of urea and creatinine in the serum.[29] Therefore, an increase in the level of urea due to extract treatment in this study also suggests some evidence of renal toxicity in experimental animals. Rhiouani and co-workers, in their studies on sub-chronic toxicity of Herniaria glabra in rodents, also reported that only the highest dose of extract results in a significant rise in the liver enzymes.[30] The findings of the present investigation are further supported by the findings of liver and kidney histological analysis of animals which also showed some histological alterations in the liver and kidney tissues of 800 mg/kg dose treated animals.

Analysis of blood parameters is also very relevant to risk evaluation, as the haematopoietic system is sensitive to toxic substances.[31] The findings of the present study revealed that highest dose of *A. calamus* rhizome extract also causes significant increase in lymphocytes and a slight decrease in WBC count. The above findings indicate that the extract also induce some toxic effects on the haematological parameters of animals. In a related study, the sub-chronic toxicity analysis of H. glabra leaves in rodents also produced some similar adverse effects on the haematological parameters of animals.[30]

The liver is one of the most important organs in the metabolism of drugs and hence histological abnormalities in liver tissues can also be correlated with biochemical results to further confirm any changes in the target organ. In the present study, the histopathology study of liver and kidney of *A. calamus* (800 mg/kg) extract treated animals further corroborate evidence of hepato-renal toxicity reflected in haematological and biochemical parameters. The histological analysis of the liver of mice treated orally with 800 mg/kg of *A. calamus* rhizome extract showed dilation of central vein indicating backflow of circulation, leucocytic infiltration and nuclear pyknosis, whereas, the histology of kidney revealed dilatation of sub-capsular space of glomeruli and distorted renal tubules. The findings of the present study on histopathological effects of *A. calamus* on liver and kidney of experimental animals are in agreement with a similar study on sub-acute treatment of H. glabra extract in rodents (4 g/kg), which showed marked centrolobular sinusoidal congestion, disruption of the central vein, hepatocellular necrosis and other significant alterations in the histo-architecture of kidney.[30] Likewise, the histopathological examination of the kidney of rats treated with Tithonia diversifolia extract also showed a dose-dependent patchy tubular necrosis in the experimental animals.[32]

In vivo genotoxicity study is an essential and obligatory component of safety-assessment programs which create a baseline of reporting requirements for evaluating the safety of herbal products.[33] In the genotoxic assessment, treatment with 800 mg/kg dose of extract revealed maximum frequency of chromosomal aberrations. Unlike in the present study, the genotoxicity investigation of Curcuma longa essential oil (1 g/kg for 14 days) was not found to produce any chromosomal aberrations in rats, which confirmed the absence of genotoxicity in this plant.[34] But, in agreement to our findings, the *in vivo* genotoxic assessment of Annona squamosa seeds extract in rats also showed similar induction of chromosomal aberrations in treated animals.[35]

In conclusion, the findings of the present study indicate several evidences of adverse effects in animals following administration of 800 mg/kg dose of *A. calamus* rhizome extract to mice. The major changes were seen in ALT, AST and total bilirubin. In addition, the histological analysis of liver and kidney presented evidences of many adverse effects at the ultrastructural level. The extract appeared significantly genotoxic at 800 mg/kg dose and induced many forms of chromosomal aberrations in mice. This work thus contributes towards the safety credentials of this plant. The use of *A. calamus* rhizomes in folk medicine should therefore be considered in the light of various inherent risks that are reflected here in this communication in its *in vivo* testing in mice.

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